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Published in:
Brain

DOI:
[10.1093/brain/awl279](https://doi.org/10.1093/brain/awl279)

Publication date:
2006

Document Version
Publisher's PDF, also known as Version of record

[Link to publication in ResearchOnline](#)

Citation for published version (Harvard):
Hye, A, Lynham, S, Thambisetty, M, Causevic, M, Campbell, J, Byers, HL, Hooper, C, Rijdsdijk, F, Tabrizi, SJ, Banner, S, Shaw, CE, Foy, C, Poppe, M, Archer, N, Hamilton, G, Powell, J, Brown, RG, Sham, P, Ward, M & Lovestone, S 2006, 'Proteome-based plasma biomarkers for Alzheimer's disease', *Brain*, vol. 129, no. Pt 11, pp. 3042-3050. <https://doi.org/10.1093/brain/awl279>

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Proteome-based plasma biomarkers for Alzheimer's disease

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Alzheimer's disease is a common and devastating disease for which there is no readily available biomarker to aid diagnosis or to monitor disease progression. Biomarkers have been sought in CSF but no previous study has used two-dimensional gel electrophoresis coupled with mass spectrometry to seek biomarkers in peripheral tissue. We performed a case-control study of plasma using this proteomics approach to identify proteins that differ in the disease state relative to aged controls. For discovery-phase proteomics analysis, 50 people with Alzheimer's dementia were recruited through secondary services and 50 normal elderly controls through primary care. For validation purposes a total of 511 subjects with Alzheimer's disease and other neurodegenerative diseases and normal elderly controls were examined. Image analysis of the protein distribution of the gels alone identifies disease cases with 56% sensitivity and 80% specificity. Mass spectrometric analysis of the changes observed in two-dimensional electrophoresis identified a number of proteins previously implicated in the disease pathology, including complement factor H (CFH) precursor and α -2-macroglobulin (α -₂M). Using semi-quantitative immunoblotting, the elevation of CFH and α -₂M was shown to be specific for Alzheimer's disease and to correlate with disease severity although alternative assays would be necessary to improve sensitivity and specificity. These findings suggest that blood may be a rich source for biomarkers of Alzheimer's disease and that CFH, together with other proteins such as α -₂M may be a specific markers of this illness.

Keywords: biomarkers; two-dimensional gel electrophoresis (2-DGE); plasma; Alzheimer's disease; proteomics

Abbreviations: AMD = age-related macular degeneration; α -₂M = α -2-macroglobulin; CFH = complement factor H; FDR = false discovery rate; 2-DGE = two-dimensional gel electrophoresis

Received July 31, 2006. Revised August 23, 2006. Accepted September 1, 2006

Background

Alzheimer's disease is the most common dementing illness of late life, affecting >20% of those aged over 80 years. Diagnosis is made primarily on clinical grounds and, to date, there is no reliable diagnostic test. With the emergence of disease modification therapies there is an increasing need for diagnostic markers to ensure that therapies are targeted at the correct patient population, to initiate early treatment and to monitor disease course.

Several studies have investigated biochemical biomarkers for this disease in various tissues including blood (Zhang *et al.*, 2004) and CSF (Hampel *et al.*, 2004). Of these

approaches, the most reproduced findings are of decreased A β ₄₂ and increased total tau and phospho-tau in CSF in Alzheimer's disease relative to normal ageing and non-dementia late life neuropsychiatric conditions (Blennow and Hampel, 2003; Sunderland *et al.*, 2005). However, lumbar puncture is a relatively invasive procedure and obtaining CSF, especially repeatedly, on large numbers of elderly infirm individuals in the community is challenging. Blood, on the other hand, is easy to obtain and as ~500 ml of CSF are absorbed into the blood every day, plasma may offer a rich source of disease biomarkers. Furthermore damage to the blood-brain barrier, a known event in this disease, may

enhance movement of proteins between brain and blood, in either direction (Zipser *et al.*, 2006). We have therefore sought to identify diagnostic biomarkers in peripheral venous blood using proteomics. Samples from patients and elderly controls were profiled by two-dimensional gel electrophoresis (2-DGE) and proteins identified by mass spectrometry and further validated by immunoblot in Alzheimer's disease and in other neurodegenerative disorders relative to controls.

Material and methods

Subjects and samples

The main study population was derived from a largely community based population of subjects with Alzheimer's disease and elderly people [Alzheimer's Research Trust (ART) cohort]. Community or nursing home resident cases with NINCDS-ADRDA probable Alzheimer's disease were identified from secondary care services for elderly people with dementia. In addition to a clinical diagnosis, subjects were assessed with a standardized assessment protocol including informant interview for diagnosis and the MMSE and Global Dementia Scale assessments for severity. Controls with an MMSE score of >26 were recruited through primary care age-sex registers or as spouses of cases. Samples were collected and prepared as described in Supplementary Material. Subjects with other neurodegenerative diseases were recruited through clinics at King's College Hospital and The Institute of Neurology, London. Samples were collected and stored as described in Supplementary Material, but in brief all samples were collected into EDTA coated glass tubes and stored for up to 2 years at -80°C . Samples from normal controls, disease controls and cases were stored under similar conditions.

Proteomics

Non-depleted plasma samples were analysed by isoelectric focusing in the first dimension on immobilized pH (3–10) gradient strips followed by PAGE in the second dimension. We chose, in this study, not to deplete the samples of abundant proteins because of concerns regarding non-specificity of all available depletion methods (*see Discussion*). Gels were silver stained and analysed quantitatively using the Melanie 2-D software version 3 to identify spots that were identifiable in both case and control groups. Spots that were significantly different between case and control groups were excised and analysed by liquid chromatography tandem mass spectrometry (LC/MS/MS). For validation plasma was analysed by western blot with complement factor H (CFH) or α -2-macroglobulin (α -₂M) primary antibodies. Full details of all methods are in Supplementary Material.

Statistics

Statistical analysis was performed using SPSS (v11), Genespring (Silicon Genetics, Redwood City, CA), and STATA (v8) packages. The mean integrated optical densities of spots on 2D gels in the initial test and replication sets were analysed using Student's *t*-test. In the full sample integrated optical densities were tested for normality using the Shapiro–Wilk test. Spots that we examined were not normally distributed and so we therefore used non-parametric tests (Mann–Whitney) combined with false discovery rate (FDR) analysis as correction for multiple comparison testing. Using the FDR as a criterion for further study by mass spectrometry

allowed us to avoid making *a priori* decisions about the significance (in terms of fold change) of spots between cases and controls. For correlation analyses individual protein integrated optical density data were first scaled to unit variance so as to standardize the scales upon which the variables were compared (i.e. each value was divided by the standard deviation of all the values for that particular variable). We then calculated the Pearson correlation coefficient. Cases with missing values were excluded pairwise. Other statistical tests are described in the text.

Results

Subject characteristics

In the core proteomics sample set of 100 subjects, cases with Alzheimer's disease were an average of 6.8 years older than control subjects and were more likely to carry one or two APOE ϵ 4 alleles (Table 1). For validation purposes three independent sets of subjects were used – from the ART cohort 266 subjects (146 controls, 111 with Alzheimer's disease, 9 with vascular dementia), from the KCL motor neuron disease (MND) study cohort 175 subjects (43 controls, 100 with MND, 18 with multiple system atrophy and 14 with other neurodegenerative, non-dementia diseases including 9 with progressive supranuclear palsy), and from the Institute of Neurology Huntington's disease study 70 subjects (15 controls, 15 pre-symptomatic mutation carriers, 15 with mild Huntington's disease and 25 with moderate or severe Huntington's disease). The study was approved by the relevant research ethics committees.

2-DGE of human plasma proteins

To examine the profile of plasma proteins we compared 25 cases and 25 normal elderly control samples by 2-DGE. We identified 217 protein spots that could be successfully identified on silver staining and matched in cases and controls (Fig. 1). We compared the mean integrated optical density (a compound measure of intensity and size) of each spot and found 15 that were significantly different between cases and controls ($P < 0.05$; *t*-test variances not assumed equal). We then repeated the experiment with a separate set of 25 cases and 25 controls. In this set 27 spots showed significant ($P < 0.05$) case–control differences and of these five showed overlap—that is they were significantly different between Alzheimer's disease and controls in both the original test set and in the replication set.

Table 1 Subject characteristics

| | Subjects; proteomic study | |
|--------------------------------------|---------------------------|------------|
| | Controls | Cases |
| Number | 50 | 50 |
| Mean age (SD) | 73.6 (5.4) | 80.4 (5.8) |
| % Female | 78 | 80 |
| % APOE ϵ 4/* | 39.6 | 80 |
| % APOE ϵ 4/4 | 0 | 12 |
| Mean MMSE score (SD) | 29.0 (1.03) | 8.5 (7.4) |
| Mean GDS score (SD) | | 5.5 (1.03) |
| Mean duration of disease (years; SD) | | 7.9 (4.3) |

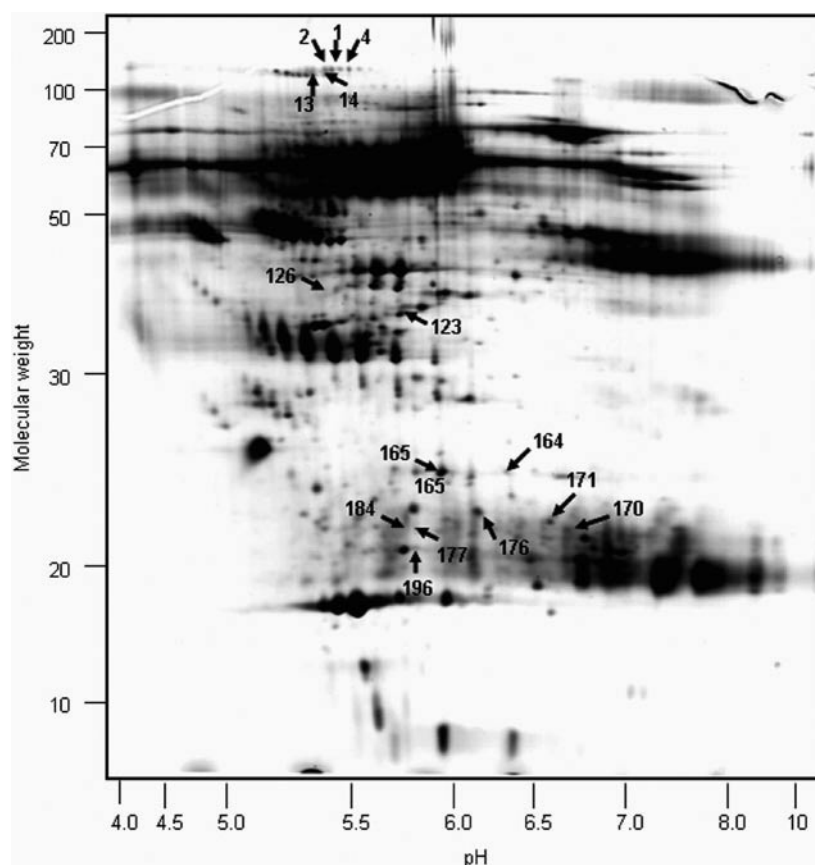


Fig. 1 Plasma protein separation. Representative two-dimensional gel electrophoresis blot of plasma. The spots showing significant differences between cases and controls (see Table 2) are labelled.

Class prediction using 2-DGE analysis

As there was reproducibility in the individual spot differences between cases and controls we then performed a class prediction analysis in order to determine whether the pattern of peptide spots on 2-DGE could predict clinical caseness. We used Support Vector Machines (SVMs), a supervised machine learning algorithm for prediction of class set in a group based upon a training set of data (Aliferis *et al.*, 2002). SVM has previously been used as a class prediction model for proteomic studies (Yang and Chou, 2004). Using GeneSpring (Silicon Genetics) we designated the original 25 cases and 25 controls as a training set and then the replication 25 cases and 25 controls as a test set. All identified proteins were used as possible predictors and with parameters polynomial dot product order 1 and diagonal scaling factor 1 we correctly identified 34 of the 50 test-samples as being either cases or controls. Sensitivity was 56% and specificity 80% using SVM analysis of 2-DGE data alone.

Identification of proteins that differentiate between cases and controls

We then compared spot density in both the initial set of cases and controls and the replication set. We assessed mean

differences between patients and controls at each spot using non-parametric tests. The *P*-values for the null hypothesis of no mean differences were saved, sorted by increasing value and ranked. A FDR index was computed and fifteen spots were identified to have a FDR of <0.50. The proteins in these fifteen spots were then identified using LC/MS/MS (Table 2).

Correlation of peptide spots with clinical parameters

The cases differed from controls by being slightly older and being more likely to carry an APOE ϵ 4 allele. It was possible that the protein differences between cases and controls could have been due to an association with these variables. We therefore performed a correlation analysis for the 15 differing spots in all subjects by age and compared spot density by APOE ϵ 4 status and gender. There were no significant differences in any spot with gender but four spots correlated with age and six spots were different in APOE ϵ 4 carriers (Table 3). As APOE genotype is itself highly correlated with Alzheimer's disease it was possible that case-control differences were entirely or largely due to protein differences in different APOE genotypes. We therefore compared spot density in cases versus controls in APOE ϵ 4 carriers only. In one case (171) the case-control difference

Table 2 Proteins identified in plasma from Alzheimer's disease and age-matched controls

| Spot No. | Rank | P-value | Fold change | State change | Protein ID | Accession no. |
|----------|------|---------|-------------|--------------|--|---------------|
| 196 | 1 | 0.0003 | 1.78 | ↑ AD | Desmoplakin | P15924 |
| | | | | | Ig kappa chain C region | P01834 |
| | | | | | Ig kappa chain V-II region TEW | P01617 |
| | | | | | Serum amyloid P-component precursor (SAP) | P02743 |
| 171 | 2 | 0.0013 | 2.11 | ↑ AD | Ig kappa chain C region | P01834 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | Galectin-7 | P47929 |
| 2 | 3 | 0.0014 | 13.75 | ↑ AD | Complement factor H (CFH) precursor | P08603 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | α ₂ -macroglobulin (α ₂ M) precursor | P01023 |
| | | | | | Ceruloplasmin precursor | P00450 |
| 184 | 4 | 0.0054 | 2.43 | ↑ AD | Ig lambda chain C regions | P01842 |
| | | | | | Ig lambda chain V-III region LOI | P80748 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | CFH related protein 2 precursor | P36980 |
| 177 | 5 | 0.0054 | 1.92 | ↑ AD | Ig lambda chain C regions | P01842 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | Ig lambda chain V-III region LOI | P80748 |
| | | | | | Ig kappa chain C region | P01834 |
| 4 | 6 | 0.0060 | 8.83 | ↑ AD | α ₂ -macroglobulin (α ₂ M) precursor | P01023 |
| 170 | 7 | 0.0117 | 1.98 | ↑ AD | Ig lambda chain C regions | P01842 |
| | | | | | Ig kappa chain C region | P01834 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | Ig lambda chain V-II region TRO | P01707 |
| | | | | | Ig kappa chain V-I region Lay | P01605 |
| | | | | | Ig kappa chain V-IV region Len | P01625 |
| 13 | 8 | 0.0155 | 4.23 | ↓ AD | Inter-alpha-trypsin inhibitor heavy chain H4 precursor | Q14624 |
| | | | | | Ceruloplasmin precursor | P00450 |
| 165 | 9 | 0.0183 | 1.58 | ↓ AD | Serum albumin precursor | P02768 |
| 164 | 10 | 0.0206 | 2.03 | ↓ AD | Complement C4 precursor | P01028 |
| | | | | | Ig gamma-1 chain C region | P01857 |
| 14 | 11 | 0.0250 | 10.82 | ↓ AD | Serum albumin precursor | P02768 |
| | | | | | Histone H2B.a/g/h/k/l | P62807 |
| 126 | 12 | 0.0290 | 1.6 | ↓ AD | CD5 antigen-like precursor | O43866 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | Ig mu chain C region | P01871 |
| 176 | 13 | 0.0291 | 1.75 | ↑ AD | Ig lambda chain C regions | P01842 |
| | | | | | Serum albumin precursor | P02768 |
| | | | | | Ig lambda chain V-III region LOI | P80748 |
| 123 | 14 | 0.0314 | 1.36 | ↑ AD | Serum albumin precursor | P02768 |
| 1 | 15 | 0.0347 | 3.32 | ↑ AD | α ₂ M precursor | P01023 |
| | | | | | Ig alpha-1 chain C region | P01876 |

was lost suggesting that this spot is a marker of APOE rather than disease status. However with all other spots the case-control difference was preserved. One spot showed a weak correlation with MMSE (spot number 4; Pearson correlation -0.3 , $P = 0.04$).

Confirmation

In order to confirm the proteomic data we analysed by western blotting the two spots containing proteins other than albumin or immunoglobulins that showed the greatest fold change between cases and controls—spot 2 containing CFH and spot 4 containing α₂M. For CFH validation we used three entirely separate sets of subjects from those used in the 2-DGE experiments; one (ART) cohort recruited as part of a study of Alzheimer's disease but also including

some patients with vascular dementia (none of these subjects overlapped with the 100 subjects used in the initial proteomics study), one cohort recruited as part of a study of MND but including some with MSA and some with PSP and other non-dementia neurodegenerative disorders, and a third cohort recruited as part of a study of Huntington's disease and including groups pre-symptomatic for the disease and those with mild or moderate/severe disease. See subject characteristics mentioned earlier.

We subjected equal volumes of plasma to immunoblot for CFH protein and subsequent quantitative densitometry (Fig. 2A). There was a highly significant difference across the nine groups [controls, Alzheimer's disease, VaD, MND, MSA, PSP and related disorders (miscellaneous in Fig. 3), pre-symptomatic Huntington's disease, mild Huntington's disease, moderate/advanced Huntington's disease; ANOVA,

Table 3 Spots showing significant differences by age or by APOE status

| Spot number | Age | | APOE $\epsilon 4/\epsilon^*$ | | |
|-------------|---------|-------|------------------------------|-------|-------|
| | Pearson | P | Difference | t | P |
| 2 | | | 0.054 | −2.78 | 0.007 |
| 13 | −0.023 | 0.02 | | | |
| 126 | −0.270 | 0.007 | −0.13 | 2.97 | 0.004 |
| 164 | | | 0.098 | −2.82 | 0.006 |
| 165 | 0.206 | 0.04 | | | |
| 170 | | | 0.13 | −3.17 | 0.002 |
| 171 | 0.205 | 0.04 | | | |
| 176 | | | 0.086 | −2.53 | 0.013 |
| 196 | | | 0.03 | −2.84 | 0.006 |

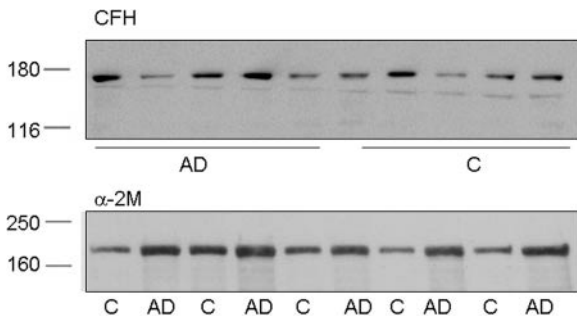


Fig. 2 Immunoblots of complement factor H and α -2-macroglobulin. The spots from 2-DGE showing most significant difference between cases and controls, identified by mass spectrometry, as CFH and α -2M and these were validated by western blotting. Representative blots are shown.

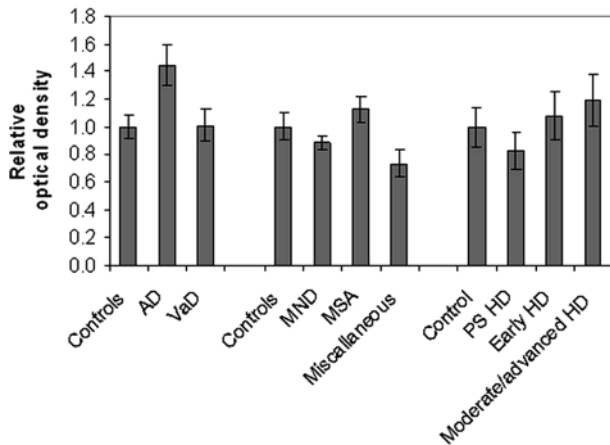


Fig. 3 Validation of change in CFH in Alzheimer's disease. Plasma from three cohorts was examined using western blotting with an antibody to CFH. Samples from disease groups were compared with control samples collected in the same studies. Significant differences between the groups were due to the elevation of CFH in the Alzheimer's disease group (ANOVA *post hoc* Alzheimer's disease-control 0.45; 95% CI 0.83–0.07; $P < 0.01$). Error bars SEM.

$F = 2.89$, $P < 0.005$]. On *post hoc* testing the only difference relative to controls was the 44.7% increase in Alzheimer's disease (Tukey's; $P < 0.01$; 95% CI 0.83–0.07; Fig. 3). In the Alzheimer's disease set there was a modest but significant

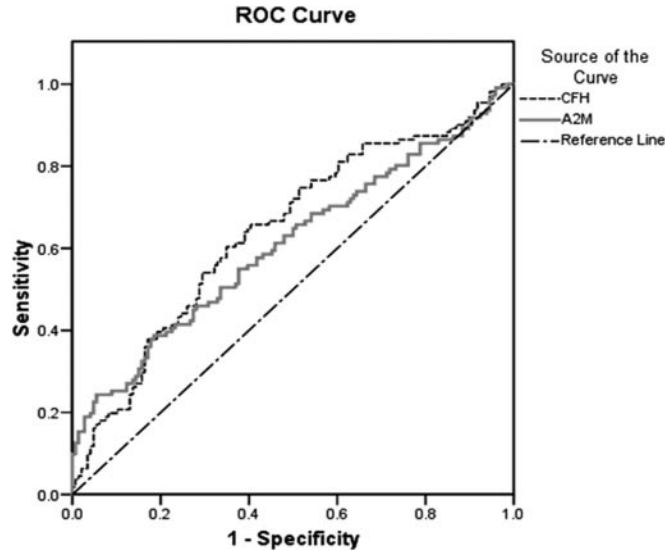


Fig. 4 Validation of change in key plasma proteins in Alzheimer's disease. ROC characteristics of α -2M and CFH. The area under the curve was highly significant (CFH 0.64, $P < 0.005$; α -2M 0.61; $P < 0.001$) but neither protein has the characteristics using this assay of a biomarker (sensitivity and specificity $> 80\%$).

correlation of CFH with MMSE (Pearson correlation coefficient -0.2 , $P < 0.05$) and a gradual (although not significant; ANOVA) increase in CFH values with global measures of severity.

We then determined α -2M levels in the dementia cohort (in total both CFH and α -2M was successfully determined in 256 subjects; Alzheimer's disease $n = 111$; controls $n = 146$; Fig. 2B). There was a small but highly significant increase in α -2M (0.99 versus 1.14; $P < 0.001$) but no significant correlation with MMSE.

Finally we performed an ROC analysis for both proteins. The area under the curve was very highly significant for both ($P < 0.005$) but neither CFH nor α -2M showed favourable test characteristics (Fig. 4). Setting specificity at 80% gave a sensitivity value of 40% for CFH and 39% for α -2M. The protein levels at these cut-offs (CFH 1.27; α -2M 1.18) were then used for a combined analysis. Combining the two proteins gave a sensitivity of 62%, specificity of 60%, a positive predictive value 54% and a negative predictive value of 68%.

Discussion

The study of human plasma proteome has become possible as highly sensitive mass spectrometry technologies have developed. Plasma is easy to obtain and contains many, only partially characterized, proteins. The amount of these proteins, or their modification, potentially reflects physiological state in normal and/or diseased conditions and may therefore be significant diagnostic markers, prognostic markers or markers of disease progression. It remains an open question whether such plasma biomarkers will be

found for Alzheimer's disease—most studies have searched for such markers in CSF, as it is more likely to reflect the metabolic state of brain (Sunderland *et al.*, 2005). However, plasma markers might exist if either plasma characteristics change in response to brain disease or if Alzheimer's disease has a systemic metabolic state that is reflected in the periphery. Both have previously been suggested (Mattila and Frey, 1995). Various candidate proteins have previously been examined in plasma and serum but no previous study has used 2-DGE based proteomic methods in a large sample set.

One of the obstacles to performing any proteomic study on plasma is the abundance of proteins such as albumin and immunoglobulins (IgG). Various strategies have been developed to deplete plasma of these proteins (Tam *et al.*, 2004) but there are at least three problems to all currently available depletion strategies and some of these are inherent to any depletion approach. First, and most problematical for any depletion of proteins, is that the high abundant targets of depletion may themselves be possible biomarkers. This is exemplified in the results of this study where some proteins shown to differ between cases and controls, including α_2 M and albumin itself, are primary targets of depletion. Indeed, one of the most promising and specific depletion techniques specifically targets a protein, α_1 -antichymotrypsin, previously shown to be a potential biomarker (DeKosky *et al.*, 2003; Zolotarjova *et al.*, 2005). Secondly, commonly used depletion strategies employed are relatively non-specific and also remove very many non-target proteins including for example CFH, one of the proteins we demonstrate to be different between cases of Alzheimer's disease, controls and other neurodegenerative diseases (Szafranski *et al.*, 2004; Zolotarjova *et al.*, 2005). Thirdly, even the most highly specific depletion strategies deplete proteins other than their primary targets and some of these could be potential biomarkers (Yocum *et al.*, 2005; Zolotarjova *et al.*, 2005). Thus for example the highly specific multiple affinity removal column (Szafranski *et al.*, 2004), although more specific than dye-based systems, also removes transthyretin which in Alzheimer's disease is altered in CSF (Biroccio *et al.*, 2006) and apolipoprotein-A1 which is altered in plasma (Liu *et al.*, 2006). We concluded therefore that it is unlikely that any existing method will turn out to be the definitive approach to blood-based biomarker discovery and in this study used a non-depletion strategy, recognizing that we would 'miss' differences in proteins of the same molecular weight as the main albumin and immunoglobulin bands. Indeed some of the proteins, including α_2 M and complement C4 for example, that we show to be altered in Alzheimer's disease are depleted even by the most specific depletion strategies available (Szafranski *et al.*, 2004). In fact it is probable that both depletion and non-depletion strategies will each yield potential biomarkers, but whichever approach is used in the discovery phase the critical step is confirmation using another method. We have shown here, that we can identify proteins that differ in plasma, without depletion, between subjects with Alzheimer's disease and

controls and have both replicated and confirmed these changes in a large sample.

Using 2-DGE we detected >200 spots, each containing multiple proteins that were successfully matched between cases and controls. Of these, four protein spot differences were replicated in a second test set of cases and controls. Moreover, using a class prediction algorithm we were able to identify cases from controls based on 2-DGE image analysis alone, with a sensitivity of 56% and a specificity of 80%. These data demonstrate that there are reproducible protein differences in plasma between people with Alzheimer's disease and healthy and cognitively intact elderly controls. 2-DGE is a separation method that is most suitable for relatively high molecular weight and relatively abundant proteins and there are other approaches to investigating the proteome some of which have been successfully applied to biomarker discovery in neurodegeneration (Irizarry, 2004; Lopez *et al.*, 2005). It is likely that these methods are complementary to those we used and that discovery of all potential biomarkers will require a suite of approaches. Our findings indicate that 2-DGE is a suitable method to discover reproducible changes in plasma between cases and controls.

In order to identify the proteins differentiating Alzheimer's disease from elderly controls we aggregated the test and replication sample set data and then used non-parametric analysis combined with FDR to identify all those spots significantly different between cases and controls. Using LC/MS/MS we have identified the peptide constituents of all 15 protein spots with a FDR <0.5. Interestingly, of these, some proteins have been previously reported to show significant associations with this disease.

Of the 15 spots, 13 contained some component of immunoglobulin or serum albumin precursor, nine showing an increase and four a decrease in Alzheimer's disease. Previously, albumin and immunoglobulins have been examined in CSF and in serum/plasma as a marker of blood–brain barrier integrity with variable findings—some showing a relative increase of immunoglobulins and/or albumin in CSF but others not replicating this finding (Hampel *et al.*, 1995, 1999). In Down's syndrome with dementia, IgA and some classes of IgG but not IgM are elevated (Mehta *et al.*, 1993). More recently attention has turned to specific immunological response in Alzheimer's disease with observed elevation of autoantibodies to the A β peptide (Nath *et al.*, 2003). These findings have led to the suggestion that this is an autoimmune disease (D'Andrea, 2005).

Some of the proteins we have identified have previously been implicated in the pathology of this illness. The role of α_2 -macroglobulin (α_2 M) has been of considerable interest since it was shown that this proteinase inhibitor was present in amyloid plaques (Bauer *et al.*, 1991). This protein also has a role in regulating immune response (Armstrong and Quigley, 2001) being induced by inflammatory cytokines (Strauss *et al.*, 1992) and may be a marker of damage to

the blood–brain barrier (Cucullo *et al.*, 2003). Genetic variation in both α_2 M and its receptor LRP have been linked with Alzheimer's disease, although these associations have not been unequivocally replicated and meta-analysis is not supportive of such an association (Bertram *et al.*, 2005). Measures of total α_2 M have not previously been reported to differ between cases of Alzheimer's disease and controls. However, differences have been reported in Alzheimer's disease and controls between the different APOE genotypes (Sacchi *et al.*, 2002), perhaps because APOE and α_2 M share, and compete for, the same receptor. We can confirm this finding as spot 4—containing only α_2 M—correlates with APOE genotype in the combined sample. We have validated the 2-DGE/MS results for α_2 M using semi-quantitative western blotting. Interestingly, another proteinase inhibitor, α_1 -antichymotrypsin, has also been suggested to be a blood-based biomarker of Alzheimer's disease in a study of similar size (DeKosky *et al.*, 2003).

However, the largest change we observed was in CFH precursor. The >10-fold change initially observed was partly due to floor-effects because of the relatively insensitive and non-specific nature of 2-DGE—some individuals with low amounts of CFH scored as having no CFH containing spots on 2-DGE thus artificially increasing the difference between cases and controls. However, using specific immunoblots we were able to confirm that CFH shows a significant increase in plasma in Alzheimer's disease but not in other neurodegenerative disorders. These data therefore not only confirm that plasma markers differentiate between Alzheimer's disease and normal healthy controls but that at least some of these changes are specific to this disease and are not found in other neurodegenerative diseases. Furthermore CFH is related to severity as levels inversely correlate with cognitive decline. The increase in CFH with decline is apparent even in the relatively early stages of dementia as assessed using the Global Deterioration Scale (GDS) (Reisberg *et al.*, 1982) with an increase in CFH in stages from GDS4 through GDS7. In stages 3 the subject is deemed to be in a pre-dementia stage, stages 4 and 5 are mild–moderate dementia where the patient has cognitive impairment but relatively little impairment in basic activities of daily living. It is only in stage 7 that the patient is severely affected and requires full nursing assistance.

CFH is a critical inhibitor of activation of the alternative complement pathway [reviewed in Rodriguez *et al.* (2004)] and has previously been shown to be present in plaques in Alzheimer's disease (Strohmeyer *et al.*, 2002) although there appears to be no increase in Alzheimer's disease brain (Strohmeyer *et al.*, 2000) or CSF (Finehout *et al.*, 2005). There is a wide variation in normal plasma levels of CFH (Esparza-Gordillo *et al.*, 2004) and recently polymorphic variation in the gene encoding CFH has been strongly associated with age-related macular degeneration (AMD) (Edwards *et al.*, 2005; Hageman *et al.*, 2005; Haines *et al.*, 2005). This is interesting as there is considerable overlap between the pathologies of AMD and Alzheimer's disease.

Like Alzheimer's disease, AMD is characterized by A β deposition (Johnson *et al.*, 2002) and associated with APOE variation (Baird *et al.*, 2004). In a longitudinal study those with AMD were shown to be at increased risk for Alzheimer's disease (Klaver *et al.*, 1999). In addition to A β and APOE, the drusen of AMD contain serum amyloid p-component (Mullins *et al.*, 2000), the precursor of which was also found to be elevated in plasma in our study. Ceruloplasmin, also previously implicated in both Alzheimer's disease and AMD (Newsome *et al.*, 1986; Loeffler *et al.*, 1994), also showed differences between Alzheimer's disease and controls in our study.

Biomarker development requires at least three phases—discovery, translation to a clinically utilizable form and validation. Our study is firmly in the first of these categories. None of the methods we have employed are feasible in routine clinical practice and validation of diagnostic markers requires very large, preferably prospective studies akin to epidemiology whereas the study reported here, although large for discovery is small for validation. Furthermore we used immunoblot analysis for confirmation, an at best semi-quantitative method. In order to validate either of the proteins we have examined in this study as diagnostic biomarkers it would be necessary to develop fully quantitative assays and test these in large, prospectively examined populations. The test characteristics of any potential biomarker are related to the assay and the sensitivity and specificity we report here are unlikely to be the same for a more quantitative assay. However, it was possible that plasma based biomarkers would not be feasible in a brain disease such as Alzheimer's disease or that any changes in plasma would be secondary to systemic illness and not specific to a particular disease. Using a proteomic approach we have shown that this null hypothesis is false, and have discovered changes in Alzheimer's disease, confirmed these in a replication proteomic study and validated in a larger data set using a complementary approach.

In summary, using a 2-DGE proteomics based approach in Alzheimer's disease plasma we find that the overall pattern of protein spot distribution is both replicable and moderately discriminative between Alzheimer's disease cases, normal controls and other neurodegenerative diseases. We have identified 15 spots containing, in addition to albumin and immunoglobulin fragments, a total of 11 proteins that differ between cases and controls. The function of almost all of these proteins is related to immune regulation adding persuasively to the considerable body of evidence suggesting an early inflammatory process in Alzheimer's disease. Our findings suggest furthermore that blood-based biomarkers are a realistic objective in Alzheimer's disease. The specificity and sensitivity of both the 2-DGE and the immunoblot assays of CFH and α_2 M are below that required for a biomarker but these are reproducible findings and suggest that more quantitative assays based on the proteins we report here may, independently or in combination, provide a biomarker for diagnosis or to monitor disease progression.

Supplementary material

Supplementary data are available at *Brain* Online.

Acknowledgements

Supported by the Alzheimer's Research Trust and the European Commission (AddNeuroMed). MT is an Alzheimer's Society Research Fellow, SJT is a UK DoH National Clinician Scientist Fellow and CES receives funding from the Motor Neurone Disease Association. Funding to pay the Open Access publication charges for this article was provided by the Alzheimer's Research Trust.

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